Azidation Technology: From Photoaffinity labeling to Molecular Tattooing

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Introduction

Life, in the form as we know it – is inseparable from motility, the capability of a living system to perform active movement. Motility is driven by special enzymes called molecular motors, which have the ability to convert chemical energy into mechanical energy. Blebbistatin is a cell permeable, specific inhibitor of class II myosins, a group of actin based ATP-driven motor proteins responsible for various biological processes, including muscle contraction, cell migration, differentiation, blebbing and cytokinesis. Although blebbistatin is the most deeply characterized and most widely used myosin II specific inhibitor, it has several limitations: 1. its high fluorescence can interfere with fluorescence based measurements, 2. photoinstability and phototoxicity upon blue light excitation, 3. cytotoxicity even without irradiation. Besides the myosin II specificity of blebbistatin, emerging evidence indicate that blebbistatin may interact with partners other than myosin II. In order to determine all binding partners of blebbistatin, we have synthesized the azido derivative of blebbistatin, azidoblebbistatin, and applied photoaffinity labeling (PAL) for target deconvolution. In PAL, the ligand - which is usually a small bioactive molecule - is supplemented with a photoreactive group (label), then introduced into the studied system and subjected to UV irradiation. Photolysis of the label results in a highly reactive intermediate capable to form covalent bond with the nearest reactive group in the ligand's binding site. In PAL experiments, arylazide derivatives are used the most often because of their chemical stability in the absence of UV irradiation, their biological inertness and the small size of the azido group. After we proved that azidoblebbistatin has identical myosin II inhibitory properties to blebbistatin's, and azidoblebbistatin can be photocrosslinked to myosin II, we confirmed that the most specific target of blebbistatin in *Dd* cells is myosin II ($EC_{50}\approx5$ μM), although at higher inhibitor concentrations we identified several weak-binding partners as well ($EC_{50}>30$ μM). During the characterization of azidoblebbistatin, we found that unlike blebbistatin, it is not phototoxic. This observation inspired us to synthesize para-nitroblebbistatin, where the azido group was replaced by a non-photoreactive nitro group, which has similar electron withdrawing characteristics. We proved by *in vitro* and *in vivo* experiments that para-nitroblebbistatin lacks all of the adverse effect of blebbistatin (photoinstability, fluorescence, cyto- and phototoxicity) while its myosin II inhibitory properties are unaffected, providing an unrestricted alternative of blebbistatin both *in vitro* and *in vivo*. Applying azidated compounds, we proved that PAL - besides UV irradiation - can also be triggered by two-photon irradiation (2PM) and realized that this reaction can serve as a basis for a new optopharmacological drug targeting technique, which we named molecular tattooing. Molecular tattooing delivers and covalently attaches a photoreactive bioactive compound to its specific target by two-photon irradiation. The biological effect of the compound can be

confined to even subfemtoliter volumes with no systemic effects outside the targeted area. Using single cell and live animal experiments we demonstrated how molecular tattooing can be applied to localize the effect of drugs even into subcellular regions.

Aims

- Chemical synthesis of azidated bioactive compounds
- *in vitro* and *in vivo* characterization of azidoblebbistatin, the first photoinduciable myosin II inhibitor
- photocrosslink of the myosin-azidoblebbistatin complex
- Target deconvolution of blebbistatin
- Chemical synthesis of C15 nitro and chloro substituted derivatives of blebbistatin
- measure the photochemical behavior, the *in vitro* and *in vivo* myosin inhibition of para-nitroblebbistatin
- characterization of two-photon activation of azidated compounds
- Spatiotemporal targeting of myosin II in live zebrafish embryo by azidoblebbistatin
- Exploring the role of myosin II in the blebbing mechanism of M2 melanoma cells by subcellular targeting with azidoblebbistatin

Results

1. *Chemical synthesis of the aryl azido derivatives of 9 bioactive compounds.*

We have developed the synthesis of 9 azido-modified bioactive compounds including antipsychotics, COX inhibitors, sodium channel blockers, myosin inhibitors and an angiotensin 2 receptor antagonist.

2. *The aryl azido derivative of blebbistatin, azidoblebbistatin has identical myosin II inhibitory properties to that of blebbistatin both in vitro and in vivo.*

We have compared the myosin II inhibition of azidoblebbistatin and blebbistatin on the basal ATPase activities of *Dd*MD (*Dictyostelium discoideum* myosin II motor domain), on the growth of *Dd* (*Dictyostelium discoideum*) cells in suspension culture and on the migration of zebrafish's pLLp (posterior lateral line primordium). In every cases we observed identical myosin inhibition by the two inhibitors.

3. *Azidoblebbistatin can be photocrosslinked to myosin resulting in a covalently bound and inhibited enzyme-inhibtor complex.*

Azidoblebbistatin was photocrosslinked to myosin in myosin enriched cell lysate while the existence of the covalently bound enzyme-inhibtor complex was detected by MS. Additionally, it was demonstrated that myosin can be covalently saturated by low concentrations of azidoblebbistatin using the technique of sequential crosslinking (subsequent azidobelbbistatin addition and UV irradiation cycles).

4. The most specific target of blebbistatin in Dd cells is myosin II (EC50≈5 μM) although at higher inhibitor concentrations (>30 μM) blebbistatin has several weak binding partners.

We prepared Dd whole cell lysate as well as myosin-enriched fractions of the lysate of a *Dd* cell line expressing recombinant *Dd*Md, and subjected the samples to increasing concentrations of azidoblebbistatin and UV irradiation. Samples were analyzed by SDS-PAGE by utilizing the fluorescence signal of azidoblebbistatin. We observed six distinct fluorescent bands in the myosin-enriched fractions, which were analyzed by densitometry and mass spectrometry. The analysis showed that azidoblebbistatin binds most specifically to myosin 2 heavy chain (EC50 = 5.1 ± 1.4 μM) and its degradation product (EC50 = 9.3 ± 3.7 μM) as well as DdMd (EC50 = 5.2 ± 0.8 μM). Further, previously unknown low-affinity (EC50 ≥ 30 μM) interacting partners of azidoblebbistatin were also identified.

5. *The para-position of the phenyl ring of blebbistatin (C15) can be modified by electron withdrawing substituents without affecting the myosin inhibitory properties of the derivatives.*

We have synthesized C15 substituted azido, nitro and chloro derivatives of blebbistatin and measured their *in vitro* and *in vivo* myosin inhibition. Based on our results we concluded that the C15 position of blebbistatin tolerates modifications.

6. Para-nitro substituted blebbistatin, para-nitroblebbistatin is photostable, non-fluorescent, non-cytotoxic and non-phototoxic.

The C15 nitro substitution of blebbistatin greatly reduces its fluorescence, improves its photostability, eliminates its cyto- and phototxicity without effecting the myosin II inhibitory properties of the molecule. Thus, para-nitroblebbistatin serves as an unrestricted and complete replacement of blebbistatin both *in vitro* and *in vivo*.

7. *Myosin II is required for scission, the final step of cell division.*

The non-phototoxicity of para-nitroblebbistatin enables fluorescent microscopic imaging of living organisms providing novel insights into the role of myosin IIs in vivo. When we time-lapse imaged HeLa Kyoto cells treated with para-nitroblebbistatin at low concentrations (<30 μM), we observed a hitherto undescribed phenotype in HeLa cells: after anaphase, the cytokinesis proceeded, the midbody formed but scission did not occur. After a while the cell collapsed and became binuclear (mechanism 1). We also found that treatment with high concentrations (>30 μM) of para-nitroblebbistatin inhibited the ingression of the cleavage furrow (mechanism 2) in which case cytokinesis did not occur. However, according to our results, para-nitroblebbistatin is not specific at high concentrations which indicates that only mechanism 1 can be attributed to the inhibition of myosin II. Consequently, scission - the final stage of cell division - requires the activity of myosin IIs. The significance of the observation is highlighted by the fact that no myosin II involvement has been reported in scission yet.

8. 2P induced photocrosslinking reaction of aryl azido derivatives of bioactive compounds can be used for their effect confinement with subfemtoliter resolution without any observable systemic effects outside the targeted area.

We demonstrated that using photo-crosslinking cycles, azidoblebbistatin can covalently saturate its target enzyme, myosin II, even at non-saturating concentrations of azidoblebbistatin. Furthermore, we revealed that photoactivation can be initiated by two-photon excitation, enabling the localization of the effect into subfemtoliter volumes. Based on these two observation we realized that 2P induced sequential crosslinking can be used for effect confinement of azidated bioactive compounds: if a whole organism is treated with low concentrations of an azidated bioactive compound (i.e. [enzyme inhibitor] << EC50), its specific effect cannot be observed. However, if a defined area (i.e. a cell or subcellular region) is illuminated continuously by two-photon irradiation, it results in the local saturation of the target enzyme as a consequence of two phenomena: covalent attachment of the azidated ligand to its target and the continuous diffusion of the unreacted azidated ligand molecules from the environment into the site of irradiation (Figure 1).

9. Bleb expansion in M2 melanoma cells is driven by globally uniform pressure while bleb retraction requires local myosin 2 activity.

Myosin 2 activity was inhibited subcellularly in blebbing M2 melanoma cells by subcellular molecular tattooing, revealing that bleb expansion is driven by globally uniform hydrostatic pressure while local myosin 2 activity is solely needed for the retraction of blebs.

Conclusions

- the chemical synthesis of azidated drugs can be efficiently performed using iodinated precursors and halogen azide exchange reactions. The synthesis is greatly aided by the fact that in case of many pharmaceutical agents the synthetic route of their iodo derivative has been explored due to the need of SAR (Structure Activity Relationship), radiolabeling for receptor binding assays, ADME (Absorption, Distribution, Metabolism, and Excretion) or SPECT (Single Photon Emission Computed Tomography) imaging.
- photocrosslinking of azidated compounds can be used as an efficient technological solution for interactome profiling, adequate for the experimental determination of the apparent binding constants of the strong as well as weak-binding protein-ligand complexes.
- C15 nitro substitution of blebbistatin eliminates the unwanted effects of the inhibitor without altering its myosin II inhibition. The unaffected inhibitory properties of para-nitroblebbistatin can be explained by the analysis of the crystal structure of the myosin.ADP.Vi.blebbistatin complex (PDB: 1YV3): the C15 nitro substitution of blebbistatin does not affect significantly the interaction of the inhibitor with myosin since the substituted group causes no steric hindrance with any of the side chains in the binding site (Figure 1).

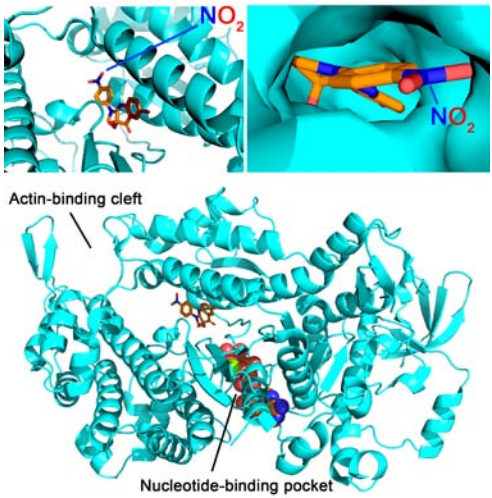

Figure 1. Structural model of the myosin.ADP.Vi.para-nitroblebbistatin complex.

- Two-photon induced photoaffinity labeling reaction can be used for spatiotemporal *in vivo* effect confinement of azidated bioactive compounds. This technology, what we named molecular tattooing, enables the investigation of local and global molecular mechanisms, autonomous or non-autonomous cellular processes by localizing the effect of bioactive compounds to even subfemtoliter volumes.

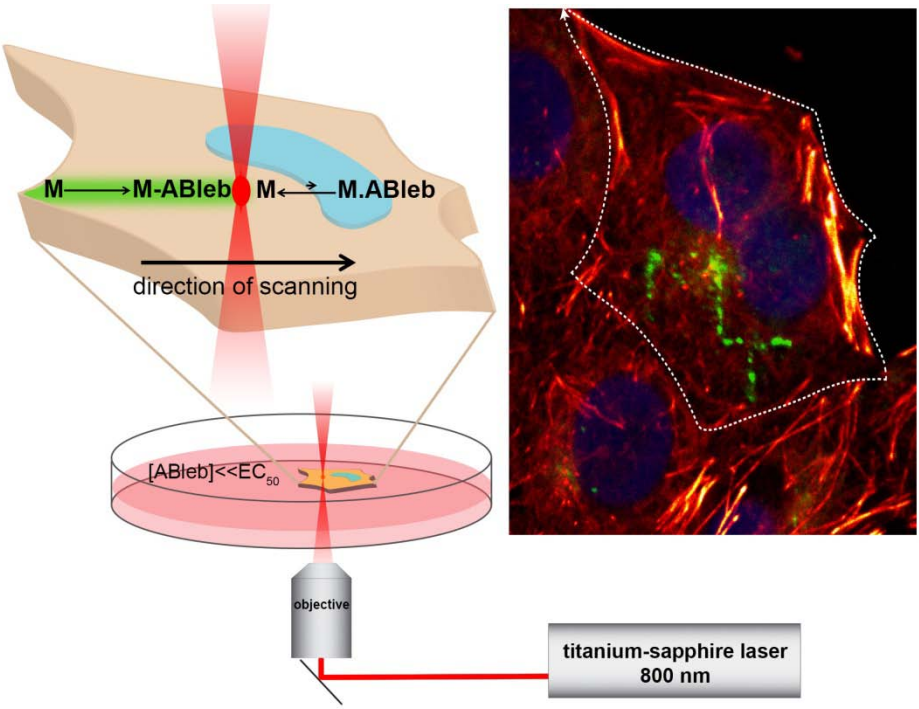

Figure 2. The concept of molecular tattooing. Azidated compounds - i.e. azidoblebbistatin (Ableb) - are designed to preserve their biological activity. They bind to the target enzyme - i.e. Myosin II (M) - and upon irradiation the bound portion of the azidated compound covalently attaches to its target while the unbound molecules react with water and become photo-inactive. This process depletes the azido compound in the irradiated target area. Since the influx of the non-reacted, active azidated compounds into the depleted area is rapid, multiple scanning of the target area leads to the covalent saturation of the target enzyme. Thus, in molecular tattooing low concentration of the azidated compound can be applied even much below EC50 within the whole organism which guarantees the lack of drug effect in the off-target areas**.** Microscopic imaging was performed by a 2PM after tattooing of a HeLa cell with azidoblebbistatin (MT shape, green). The actin network and the nuclei were stained by actin glow (red) and Hoescht (blue), respectively, after tattooing.

Applied Techniques

- Organic chemistry: electrophilic substitutions (halogenations), copper (I) catalyzed halogen-azide exchange reactions, esterification, tosylation, asymmetric synthesis, microwave promoted synthesis
- Flash chromatography, HPLC (High Pressure Liquid Chromatography), LC-MS (Liquid Chromatography-Mass Spectrometry)
- NMR (Nuclear Magnetic Resonance Spectroscopy) - performed by Andrea Bodor
- Protein expression and purification - performed by Ozoróczyné Ilona Szász
- Absorption spectroscopy (Schimadzu UV-2101 spectrophotometer)
- Fluorescent spectroscopy (Edinburgh Instruments F900 Fluorescence Spectrometer)
- Steady-state basal and actin activated ATPase activities measurements - performed by Boglárka Várkuti and László Végner
- Gel electrophoresis
- Identification of tryptic protein fragments by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time Of Flight) mass spectrometry - performed by Éva Gulyás
- Cellular assays (*Dd*, HeLa, M2)
- Live animal experiments (zebrafish)
- Confocal microscopy (LSM 710, Zeiss)
- Two-photon microscopy (Femto 2D, Femtonics)

Publications Concerning this Thesis

Miklós Képiró, Boglárka H. Várkuti, Andrea Bodor, György Hegyi, László Drahos, Mihály Kovács and András Málnási-Csizmadia. *Azidoblebbistatin, a photoreactive myosin inhibitor.* Proceedings of the National Academy of Sciences 109, no. 24 (2012): 9402-9407.

Miklós Képiró, Boglárka H. Várkuti, László Végner, Gergely Vörös, György Hegyi, Máté Varga and András Málnási-Csizmadia. *para-Nitroblebbistatin, the Non-Cytotoxic and Photostable Myosin II Inhibitor.* Angewandte Chemie, 126(31), (2014): 8350-8354.

Miklós Képiró, Boglárka H. Várkuti, Anna Rauscher, Miklós Z. Kellermayer, Máté Varga and András Málnási-Csizmadia. *Molecular tattoo: subcellular confinement of drug effects* – under submission

Conference Proceedings (the presenting author is underlined)

Molecular tattooing in live zebrafish: inhibition of myosin II dependent processes in space and time
Miklós Képiró, Boglárka H. Várkuti, Máté Varga, András Málnási-Csizmadia
2013 Barcelona, Spain, 8th European Zebrafish Meeting, oral presentation

Interactomics of blebbistatin
Miklós Képiró*, Boglárka Várkuti Zhen Hui Yang, András Málnási-Csizmadia*
2013 Philadelphia, US, Biophysical Society 57th Annual Meeting, poster presentation

Partner mapping of azidoblebbistatin, the novel photo-inducible myosin inhibitor
Miklós Képiró*, Boglárka H. Várkuti, György Hegyi, Mihály Kovács, András Málnási-Csizmadia*
2012 Rhodes, European Muscle Conference, poster presentation

Structural Model of the Pre-Powerstroke State of the Actomyosin Complex
Zhen Hui Yang, Boglárka H. Várkuti, Anna Rauscher, ***Miklós Képiró****, András Málnási-Csizmadia*
2012 San Diego, US, Biophysical Society 56th Annual Meeting, oral presentation

Synthesis and functional characterization of azido-blebbistatin, a photoreactive myosin inhibitor
Miklós Képiró*, Boglárka H. Várkuti, György Hegyi, Mihály Kovács, András Málnási-Csizmadia*

Biophysical Society 56th Annual Meeting, 2012 San Diego, US, Poster presentation

Synthesis and functional characterization of azido-blebbistatin, a photoreactive myosin inhibitor
Miklós Képiró*, Boglárka H. Várkuti, György Hegyi, Mihály Kovács, András Málnási-Csizmadia*
2011 Berlin, European Muscle Conference, oral presentation

Sythesis and functional characterization of azido-blebbbistatin, a photoreactive myosin inhibitor
Miklós Képiró*, Boglárka Várkuti and András Málnási-Csizmadia*
2011 Budapest, Hungary, 4th European Conference on Chemistry for Life Siences, poster presentation

Other Publications

Boglárka H. Várkuti and **Miklós Képiró**. *Hatékonyabb gyógyszerek, de hogyan? Kiút a kutatás válságából*. Élet és tudomány, ISSN 0013-6077, 2014. (69. évf.), 35. sz., 1094-1096. p.

Miklós Képiró and András Málnási-Csizmadia. *Hatóanyagok in vitro tesztelése.* Budapest: Moravcsik Alapítvány, 2011. pp. 22-35. (ISBN:978-963-89479-0-1). Book chapter

Gabriella Felföldi, Judit Marokházi, **Miklós Képiró**, István Venekei. *Identification of natural target proteins indicates functions of a serralysin-type metalloprotease, PrtA, in anti-immune mechanisms***.** Applied and environmental microbiology, 2009, 75.10: 3120-3126.